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ATP7B activity is stimulated by PKCε in porcine liver

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ABSTRACT

Copper is necessary for all organisms since it acts as a cofactor in different enzymes, although toxic at high concentrations. ATP7B is one of two copper-transporting ATPases in humans, its vital role being manifested in Wilson disease due to a mutation in the gene that encodes this pump. Our objective has been to determine whether pathways involving protein kinase C (PKC) modulate ATP7B activity. Different isoforms of PKC (α, ε, ζ) were found in Golgi-enriched membrane fractions obtained from porcine liver. Cu(I)–ATPase activity was assessed in the presence of different activators and inhibitors of PKC signaling pathways. PMA (10^{-8} M), a PKC activator, increased Cu(I)–ATPase activity by 60%, whereas calphostin C and U73122 (PKC and PLC inhibitors, respectively) decreased the activity by 40%. Addition of phosphatase λ decreased activity by 60%, irrespective of pre-incubation with PMA. No changes were detected with 2 μM Ca^{2+}, whereas PMA plus EGTA increased activity. This enhanced activity elicited by PMA decreased with a specific inhibitor of PKCε to levels comparable with those found after phosphatase λ treatment, showing that the ε isoform is essential for activation of the enzyme. This regulatory phosphorylation enhanced V_{max} without modifying affinities for ATP and copper. It can be concluded that signaling pathways leading to DAG formation and PKCε activation stimulate the active transport of copper by ATP7B, thus evidencing a central role for this specific kinase-mediated mechanism in hepatic copper handling.

1. Introduction

Physiologically, copper is an essential heavy metal for almost all organisms, being an important cofactor for many different proteins. However, an excess can also be very toxic, producing harmful reactive oxygen species (Gupta and Lutsenko, 2009). Therefore, intracellular copper concentration and handling are precisely controlled by different copper-binding proteins. In mammals, two proteins are responsible for active copper transport in the Golgi network, Cu(I)–ATPases ATP7A and ATP7B, products of the genes *ATP7A* (OMIM 300011) and *ATP7B* (OMIM 606882). Specific mutations in these genes cause two different disorders in human copper homeostasis, the Menkes and Wilson diseases. The past three decades have seen characterizations of the catalytic cycle, structure, localization and copper affinity of Cu(I)–ATPases from bacteria to humans (Lutsenko et al., 2007). Only in recent years has the importance of post-translational regulation mediated by protein kinases emerged, demonstrating that these enzymes regulate different Cu(I)–ATPases at the protein expression level and its trafficking (Vanderwerf et al., 2001; Pilankatta et al., 2011; Veldhuis et al., 2009), copper-binding affinity (Veldhuis et al., 2011), and the catalytic cycle (Valverde et al., 2008, 2011; Hilário-Souza et al., 2011). However, in most cases the protein kinases involved are unknown. Almost all these studies used heterologous expression and/or overexpression of a Cu(I)–ATPase. Therefore, major advances in understanding the molecular mechanism by which protein kinases specifically regulate Cu(I)–ATPase activities could be achieved by investigating copper pumps in their native membrane environments.

We have previously demonstrated that PKA is responsible for modulating the activities of different Cu(I)–ATPases (Valverde et al., 2008, 2011; Hilário-Souza et al., 2011), indicating that kinase-mediated phosphorylation is a key mechanism in the physiological regulation of active copper transport. It is conceivable that other kinases are involved, but no description of the regulation of

Abbreviations: BCS, bathocuproine disulfonate; MOPS, 3-(*N*-morpholino) propanesulfonic acid; PMA, phorbol 12-myristate 13-acetate; PPase λ, protein phosphatase λ.

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Cu(I)–ATPase by protein kinase C (PKC) seems to be carried out. PKC is a superfamily of serine/threonine kinases involved in many different signaling events. Distinct isoforms are grouped into three subfamilies: (i) classical (α, βI, βII, γ), which are activated by Ca^{2+}, diacylglycerol (DAG) and phospholipids; (ii) novel (δ, ε, η, θ), which are regulated by DAG and phospholipids, but are independent of Ca^{2+}; and (iii) atypical (ζ,ι/λ), which do not respond to either Ca^{2+} or DAG, but are activated by other lipids, protein-protein interactions or phosphorylation (reviewed by Parker and Murray-Rust, 2004).

We have investigated whether the PLC → PKC signaling pathway modulates porcine liver Cu(I)-ATPase activity. Among the different PKC isoforms detected in liver, we found that PKCε has a key regulatory role in activating native ATP7B in its natural membrane moiety.

2. Materials and methods

2.1. Reagents

Buffers, protease inhibitors, ATP, U73122, phorbol 12-myristate 13-acetate (PMA) and monoclonal antibody against β-actin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphatase λ was purchased from Millipore Co. (Billerica, MA, USA). The specific PKC isoform inhibitors and polyclonal antibodies against ATP7B and different isoforms of PKC were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Calphostin C was obtained from Calbiochem/Merck (Darmstadt, Germany). Nitrocellulose and PVDF membranes, ECL™ system and secondary antibodies were from GE Healthcare (GE Healthcare, São Paulo, Brazil). Pro-Q Diamond Phosphoprotein Blot Stain and SYPRO Ruby Protein Blot Stain were purchased from Molecular Probes Invitrogen (Eugene, OR, USA). Porcine livers were obtained from a slaughterhouse supervised by a veterinarian and under sanitary laws. All other chemical reagents were of the highest available purity.

2.2. Preparation of membrane fractions

Golgi membrane fractions were obtained as described by Hilário-Souza et al. (2011). The membrane fractions were resuspended in MOPS-KOH (pH 7.6) containing 1 mM DTT and stored in liquid N_2. Protein concentration was measured by the Phenol-reagent method (Lowry et al., 1951), using BSA as the standard.

2.3. Identification of PKC isoforms by Western blotting

Different samples (20 and 50 μg of protein) were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk, incubated overnight with the primary antibody against the αPKC isoform (1:1500) at 4 °C, incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody at room temperature, and developed with the ECL™ system. After stripping (0.2 M glycine, pH 2.2), the membrane was used to immunodetect other PKC isoforms (λ, ε and ζ; 1:500 dilution). Control loading was carried out using the monoclonal antibody against β-actin (1:5000).

2.4. Cu(I)–ATPase activity

Activity was assayed using 0.25 mg/ml protein (in membrane fractions) incubated for 30 min on ice under two different conditions: (a) assay medium with 100 μM ascorbate and 1 mM Na_2SO_3, and (b) assay medium with 250 μM BCS – a copper chelator – without the reducing agents. After equilibration at 37 °C, the reaction was started by adding 5 mM ATP (Hilário-Souza et al., 2011). Cu(I)–ATPase activity was measured as the difference between P_i released under conditions (a) and (b). Reactions were stopped after 5 min with TCA (5% w/v), and ammonium molybdate and P_i was measured after adding the reducing agent (Fiske and SubbaRow, 1925). Specific additions aimed to activate or inhibit PKC are indicated in the corresponding figure legends.

2.5. Influence of PKC pathway modulators on Cu(I)–ATPase activity

Modulation of Cu(I)–ATPase activity by PKC pathways was measured by ATP hydrolysis in the presence of different activators (PMA and Ca^{2+}) and inhibitors (calphostin, U73122, iPKCζ and iPKCε) of these pathways. Membrane fractions were pre-incubated for 30 min with different concentrations of PMA, calphostin C, U73122, iPKCε and iPKCζ in an ice bath. Concentrations are indicated in the corresponding figures and legends. Assays with 2 μM free Ca^{2+} or 2 mM EGTA were used to differentiate between Ca^{2+}-dependent (conventional) and -independent (novel) PKCs. The 2 μM free Ca^{2+} condition was established using $CaCl_2$ and EGTA according to Sorenson et al. (1986). In the phosphatase λ assay, the membrane fractions were pre-incubated on ice for 30 min in a medium with or without PMA. There was further 5 min incubation at 4 °C after the phosphatase was added before the reaction was initiated with ATP.

2.6. Determination of PKC signaling influence upon ATP7B enzymatic parameters

To establish whether PKC leads to changes in affinities and/or V_{max} values, ATP7B activity was measured in media containing different concentrations of ATP (at fixed copper) and copper (at fixed ATP). Since the Cu(I)–ATPase activity was measured in the presence of contaminant copper only, it was necessary to remove copper from the samples with BCS as described by Hilário-Souza et al. (2011).

2.7. Catalytic phosphorylation of ATP7B by [γ-^{32}P]ATP and decay of the phosphorylated intermediate

Catalytic phosphorylation of ATP7B was measured as described by Lowe et al. (2004). The membrane fractions (50 μg protein) were suspended in medium containing 20 mM BIS-TRIS Propane (pH 6.0), 200 mM KCl and 5 mM $MgCl_2$, and pre-incubated for 30 min on an ice bath with or without 10^{-8} M PMA. The reaction (at 0 °C) was started by adding [γ-^{32}P]ATP (final concentration 5 μM; 1500 cpm/pmol). To follow the phosphoenzyme decay, aliquots (200 μl) were removed at the times shown on the abscissa (10, 30, 60, 300 s) and mixed with 50 μl TCA 50% supplied with 1 mM NaH_2PO_4 in order to stop the reaction. These quenched samples were centrifuged for 15 min at 13,300 × *g*. The pellet was washed twice with 100 μl TCA 5% plus NaH_2PO_4 1 mM, and resuspended in 40 μl acidic sample buffer. The samples were separated by acidic electrophoresis to avoid breakdown of the acyl-phosphoprotein (Weber and Osborn, 1969) and the gels exposed to a phosphorscreen for analysis using a Phosphor Imager (Cyclone Plus, Perkin Elmer, Waltham, MA, USA). The phosphorylated bands were quantified with ImageJ software, and each gel was stained with Coomassie Blue to normalize the phosphorylated protein at 160 kDa by the total protein loaded in each lane.

2.8. Analysis of regulatory phosphorylation of ATP7B by PKC

Samples were incubated in buffer containing 20 mM MOPS-KOH (pH 7.5), 50 mM K_2SO_4, 10 mM $MgSO_4$, 4% Glycerol, 10 mM NaN_3, 10 mM NaF, 0.75 mg/ml protein. They were pre-incubated for 30 min in an ice bath with or without 10^{-8} M PMA or 10^{-8} M calphostin C. After equilibration at 30 °C, the reaction was started

by addition of ATP (final concentration 62.5 μM) and stopped after 30 min with 40 μl of SDS-PAGE sample buffer. The samples were separated by SDS-PAGE 10% (Laemmli, 1970), and transferred to PVDF and nitrocellulose membranes; note that the alkaline pH of the Laemmli's buffer preserves the regulatory phosphorylation, whereas the catalytic phosphorylated intermediate is broken. The membranes were stained with Pro-Q Diamond Phosphoprotein and total protein was measured using SYPRO Ruby Protein. An Ettan DIGE Imager (GE Healthcare) scanner analyzed the stained membranes, and the desired bands were quantified with ImageJ software. The nitrocellulose membranes were immunoblotted using primary antibody against ATP7B (1:500) and fluorochrome-conjugated secondary antibody. Membranes were analyzed using scanner Odyssey (Li-Cor Biosciences, Lincoln, NK, USA). The available commercial antibody against ATP7B was not suitable for immunoprecipitation assays using different protocols (A/G protein agarose or magnetic beads), despite the manufacturer's datasheet.

2.9. Alignment and prediction of PKC-target sites

The amino acid sequences of ATP7B from different species as sheep (Q9XT50), mouse (Q64446), human (P35670), rat (Q64535) and pig (A5A789) were aligned using the Clustal method (Larkin et al., 2007) with default parameters and analyzed *via* Jalview 2.81 (Waterhouse et al., 2009). The identifiers above indicate the UniProtKB accession numbers (Consortium, 2014). The PKC phosphorylation sites prediction was carried out using different tools, in order to maximize the confidence of prediction: Phosphosite (Hornbeck et al., 2012), Scansite 2.0 (Obenauer et al., 2003) and NetPhosK 1.0 (Blom et al., 2004).

2.10. Statistical analysis of the data

Each experiment was performed with at least four different membrane fractions and Cu(I)-ATPase activity was assayed in triplicate. When the results are presented as percent of control activity (basal condition with no addition of activators or inhibitors in replicates carried out in parallel), the SEM is calculated from absolute values (in nmol $P_i \times$ mg protein$^{-1} \times$ min^{-1}) and then converted to percentages. The data were analyzed using one-way ANOVA. The post-test analysis significance of the differences was verified by the Newman–Keuls or Bonferroni post-tests, as indicated in the figure legends (significance was set at $p < 0.05$). Asterisks or different lower-case letters above the graph bars indicate statistical differences between groups. Differences between V_{max}, K_m (ATP curve) and $K_{0.5}$ (free copper curve) values in control conditions *vs* activation, or in control conditions *vs* inhibition of PKC were assessed with Student's *t* test.

3. Results

3.1. PMA increases porcine liver ATP7B activity

PMA, an analog of the physiological diacylglycerol (DAG), an intermediate in the PLC → PKC pathway, is an activator of conventional and novel PKC isoforms (Newton and Messing, 2010). Basal Cu(I)–ATPase activity (*i.e.* without exogenous kinase activators and/or inhibitors) increased up to 60% after liver Golgi-enriched membranes had been pre-incubated with raising concentrations of PMA (from 10^{-11} to 10^{-6} M) (Fig. 1), suggesting a PKC-mediated stimulation of the hepatic ATP7B.

3.2. Calphostin C inhibits ATP7B activity

Following from the above results, membrane fractions were pre-incubated under visible light with calphostin C, which binds irreversibly to the DAG site in the regulatory domain of PKC. The basal activity of Cu(I)-ATPase decreased 40% with raising concentrations of calphostin C (from 10^{-11} to 10^{-6} M) (Fig. 2).

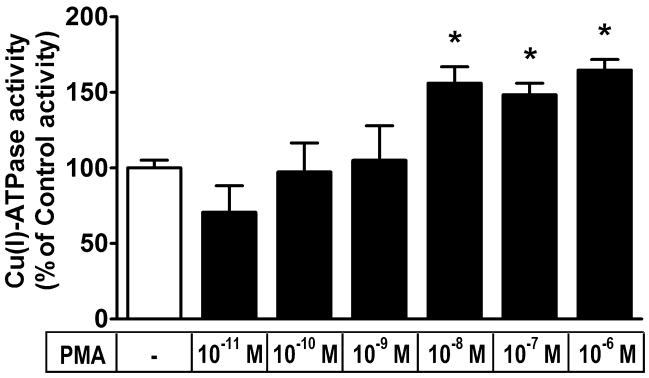

Fig. 1. Stimulation of ATP7B activity by PMA. Membrane fractions were incubated for 30 min in an ice bath with different concentrations of PMA, as indicated on the abscissa. ATP7B activity was measured as described in Materials and methods. The control bar shows the activity with no addition of PMA. Data are mean ± SEM from experiments carried out using different membrane preparations ($n = 4$). * Statistically significant different from control ($p < 0.05$) (one-way ANOVA followed by Bonferroni post-test).

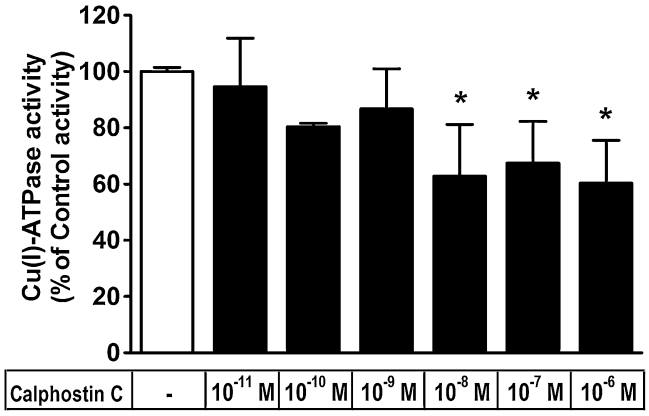

Fig. 2. Inhibition of ATP7B activity by calphostin C. Membrane fractions were incubated for 30 min in an ice bath with different concentrations of calphostin C, as indicated on the abscissa. ATP7B activity was measured as described in Materials and methods. The control bar shows the activity with no addition of calphostin C. Data are mean ± SEM from experiments carried out using different membrane preparations ($n = 4$). * Statistically significant different from control ($p < 0.05$) (one-way ANOVA followed by Bonferroni post-test).

3.3. Phosphatase λ abolishes the stimulatory effect of PMA on ATP7B activity

Phosphatase λ was added to remove phosphoryl groups from serine/threonine residues. ATP7B activity decreased by 60% (Fig. 3, third column), indicating that the basal activity was already under kinase-mediated phosphorylation(s), which can modulate the copper pump. When phosphatase λ was added after the membranes had been incubated with PMA, ATP7B activity dropped to the same lower values (Fig. 3, last column), giving support to the view that PMA-induced activation of the copper pump relies on a PKC-mediated mechanism.

3.4. PLC is involved in the activation of ATP7B by DAG

To investigate whether PLC-mediated generation of DAG is involved in the signaling pathway that culminates in increased ATP7B activity, different concentrations of the PLC inhibitor

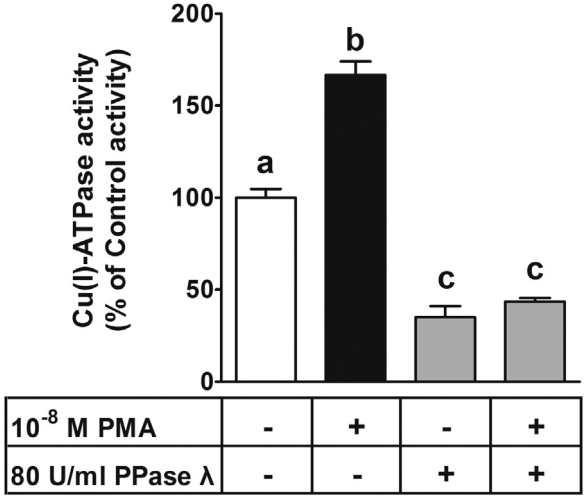

Fig. 3. Phosphatase λ inhibits basal ATP7B activity and abolishes the stimulatory effect of PMA. ATP7B activity was measured in the absence of additions, and in the presence of PMA and phosphatase λ in the combinations shown on the abscissa. After 30 min pre-incubation in the presence or absence of PMA, phosphatase λ was added to the reaction medium for 5 min before the reaction was started by ATP addition. Data are mean ± SEM ($n = 4$). Different lower-case letters above the bars indicate statistically significant differences ($p < 0.05$) (one-way ANOVA followed by Newman–Keuls post-test).

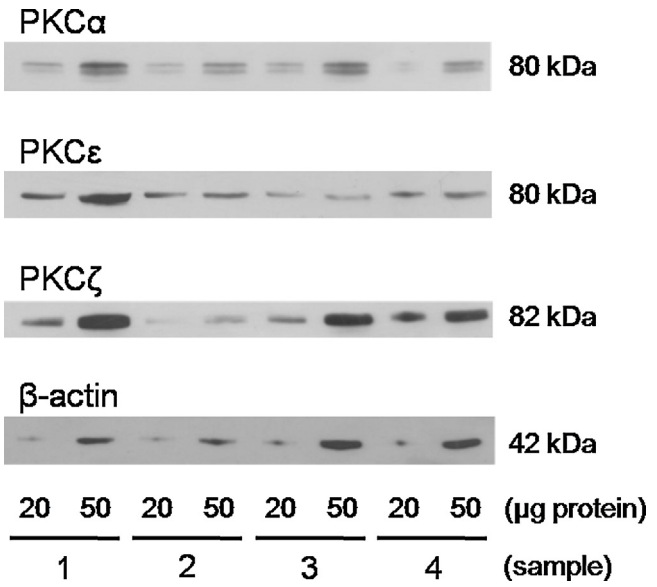

Fig. 5. Identification of different isoforms of PKC in Golgi-enriched liver membrane fractions. Membrane-associated PKC isoforms were detected by immunoblotting with specific antibodies against the α, ε and ζ isoforms. Four different membranes fractions were analyzed using two amounts of total protein (20 and 50 μg per lane), as indicated at the bottom of the figure. Blots were stripped and reprobed for β-actin as the loading control.

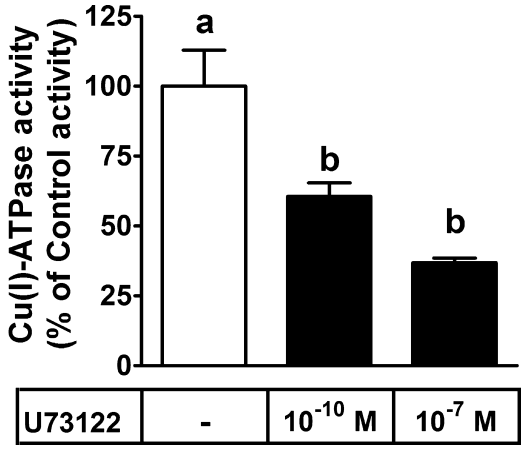

Fig. 4. PLC is involved in the activation of ATP7B by DAG. ATP7B activity was measured after 30 min pre-incubation with the different concentrations of U73122 indicated on the abscissa. Data are mean ± SEM ($n = 4$). Different lower-case letters above the bars indicate statistically significant differences ($p < 0.05$) (one-way ANOVA followed by Newman–Keuls post-test).

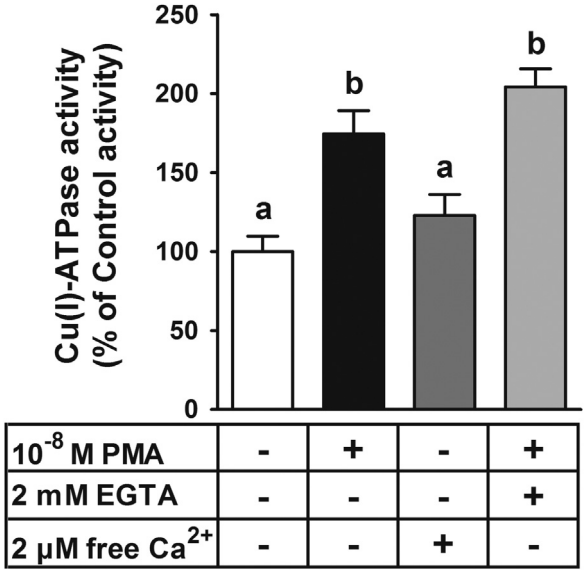

Fig. 6. ATP7B activity is modulated by a PKC from the novel family. Membrane fractions were pre-incubated with 10^{-8} M PMA, 2 μM free Ca^{2+} and 2 mM EGTA in the combinations shown on the abscissa. Data are mean ± SEM ($n = 4$). Different lower-case letters above the bars indicate statistically significant differences ($p < 0.05$) (one-way ANOVA followed by Newman–Keuls post-test).

U73122 were tested. The inhibitor decreased Cu(I)-ATPase activity at sub-nanomolar concentrations (Fig. 4), which confirms DAG production by PLC being associated with PKC-mediated stimulation of ATP7B. It also demonstrates that a membrane-associated PLC is already active and contributes to the basal Cu(I)-ATPase activity.

3.5. Identification of different PKC isoforms in the Golgi membranes

Western blotting with specific antibodies was used to determine which are the membrane-associated PKC isoforms. Isoforms from all PKC families were identified in four different membrane samples: PKCα (conventional), PKCε (novel) and PKCζ (atypical) (Fig. 5). However, PKCλ was not detected (data not shown). We therefore decided to determine which isoform family is responsible for the activation of ATP7B.

3.6. A novel isoform of PKC is responsible for stimulating ATP7B

Since ATP7B activity was stimulated by PMA, indicating the involvement of a DAG-dependent PKC, we determined whether a conventional or a novel PKC isoform is responsible for triggering the effect on ATPase activity. Only the conventional PKC isoforms are Ca^{2+} dependent. ATP7B activity was unaltered when 2 μM free Ca^{2+} (Ca·EGTA buffer) alone was added; moreover, removal of Ca^{2+} by EGTA did not modify the stimulatory effect of PMA (Fig. 6). These combined experiments show that a PKC isoform from the novel family is responsible for activating ATP7B.

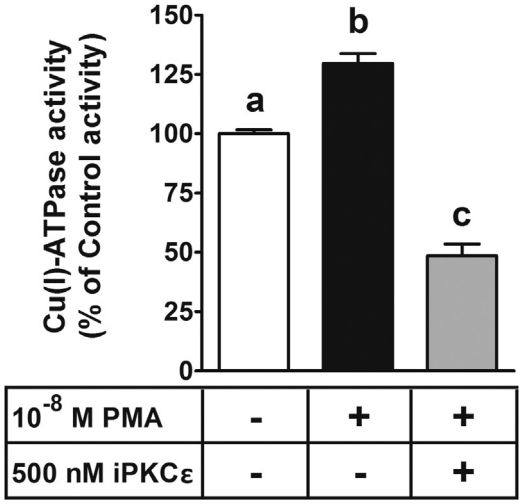

Fig. 7. PKCε is the isoform responsible for activating ATP7B. ATPase activity was measured using 500 nM of the specific inhibitor of PKCε (iPKCε) in the presence of 10^{-8} M PMA. Data are mean ± SEM ($n = 4$). Different lower-case letters above the bars indicate statistically significant differences ($p < 0.05$) (one-way ANOVA followed by Newman–Keuls post-test).

3.7. PKCε is the isoform involved in the stimulation of ATP7B activity

To confirm whether PKCε is responsible for activating the ATPase, a specific PKCε inhibitor peptide, iPKCε, was used. Pre-incubation of the membranes with iPKCε decreased PMA-activated ATP7B activity (Fig. 7) to the levels when phosphatase λ (Fig. 3) or U73122 (Fig. 4) was added. This accentuated specific inhibition proves that the PKCε isoform from the novel family is responsible for stimulating the liver ATP7B. The lack of an effect with iPKCζ (data not shown) disproves the involvement of the atypical PKCζ.

3.8. PKC signaling induces increase in ATP7B V_{max} for ATP and copper with no changes in substrate affinities

To determine which changes in enzymatic parameters were induced by PKC signaling, ATP7B activity was measured in different concentrations of ATP (fixed copper) or copper (fixed ATP). To investigate the influence of PKC stimulation at different Cu(I)–ATPase activity levels, catalysis was assayed with or without PMA in different ATP concentrations (Fig. 8). Analysis of the two curves using the Michaelis–Menten equation indicates that PMA treatment raises V_{max} (39.2 ± 5.3 nmol $P_i \times$ mg protein$^{-1} \times$ min^{-1} *vs* 24.1 ± 1.9; $p < 0.05$), whereas the K_m remains unchanged (1.2 ± 0.5 mM in control conditions *vs* 1.5 ± 0.4 in the presence of PMA). The influence of PKC inhibition was assessed at different levels of ATP7B activity attained in different concentrations of copper. The data were fitted by a Hill sigmoidal curve (Sigma Plot Software). There was no alteration in the $K_{0.5}$ for copper ($1.2 \times 10^{-17} \pm 0.03 \times 10^{-17}$ M in control conditions *vs* $1.0 \times 10^{-17} \pm 0.03 \times 10^{-17}$ M in the presence of calphostin C); as expected, V_{max} decreased from 45.4 ± 4.3 to 26.1 ± 3.6 nmol $P_i \times$ mg protein$^{-1} \times$ min^{-1} when PKC was inhibited by calphostin C (Fig. 9).

3.9. PMA and calphostin C effect on regulatory phosphorylation of ATP7B

To investigate whether the increase in Cu(I)-ATPase activity was in fact due to phosphorylation of ATP7B by PKC, assays to detect the phosphorylation levels of Ser/Thr residues at 160 kDa

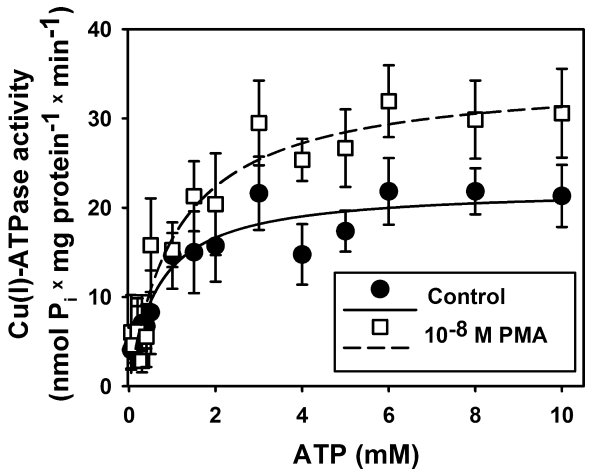

Fig. 8. PMA leads to increased V_{max} of ATP7B, but does not change K_m for ATP (at fix, contaminant copper). Cu(I)–ATPase activity was measured at different concentrations of ATP, in the absence and presence of 10^{-8} M PMA. A Michaelis–Menten curve was adjusted to the experimental points and the kinetic parameters (see text) were calculated using the values obtained in different independent fittings ($n = 4$).

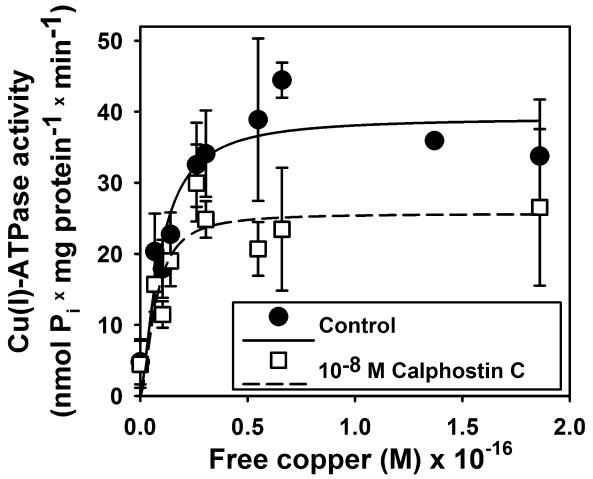

Fig. 9. Calphostin C decreases V_{max} of ATP7B, but does not change its $K_{0.5}$ for copper. Cu(I)–ATPase activity was measured in the presence of 5 mM ATP at different concentrations of free copper (BCS·Cu buffer; Hilário-Souza et al., 2011), with and without 10^{-8} M calphostin C. The data were fitted using the Hill sigmoidal equation ($v = V_{max} \times [\text{free Cu}]^n / \{(K_{0.5})^n + [\text{free Cu}]^n\}$. The kinetic parameters (see text) were calculated using the values obtained in different independent fittings ($n = 4$).

were carried out. A small, but statistically significant, increase of phosphorylation in the presence of PMA was seen when compared with the control condition, whereas calphostin C led to an equivalent decrease in the level of regulatory phosphorylation of ATP7B (Fig. 10) phosphorylation level. Taken together these data confirm that PKC modulates ATP7B activity by direct phosphorylation, a signal that is amplified downstream to achieve a robust and specific response of the pump activity in its native state within the Golgi membrane environment.

3.10. PMA-induced regulatory phosphorylation did not change catalytic phosphorylation steps

Finally, we investigated the evolution of catalytic phosphorylation of ATP7B with time after addition of [γ-^{32}P]ATP (10, 30, 60 and 300 s). The longer times (60 and 300 s) were chosen to investigate the influence of PKC-mediated regulatory phosphorylation on the decay of the catalytic phosphorylated intermediate at a low micromolar ATP concentration, i.e. in a condition of which its breakdown

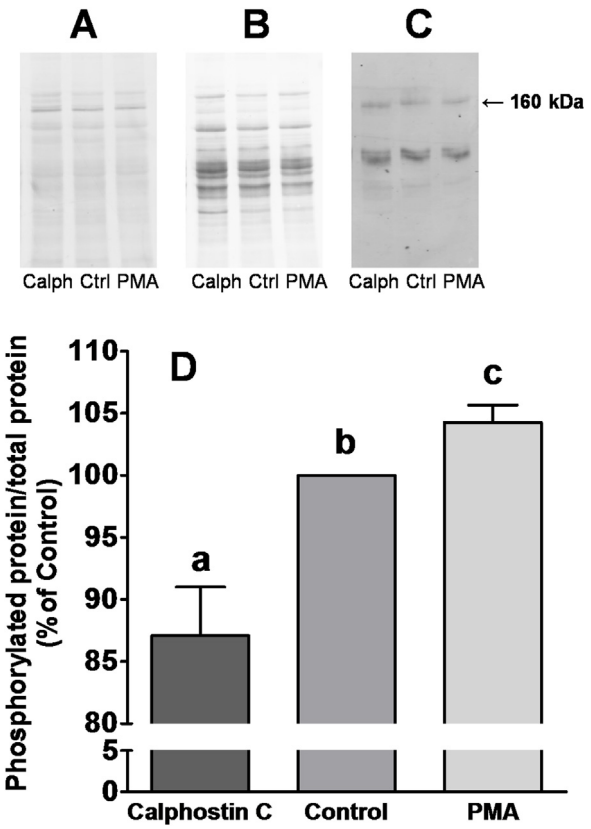

Fig. 10. PKC-mediated regulatory phosphorylation of ATP7B. Upper panels: (A) representative PVDF membrane stained with Pro-Q Diamond, in the following conditions: 10^{-8} M calphostin C (left), control (middle) and 10^{-8} M PMA (right); (B) representative PVDF membrane stained with SYPRO Ruby in the following conditions: 10^{-8} M calphostin C (left), control (middle) and 10^{-8} M PMA (right); (C) representative nitrocellulose membrane immunoblotted against ATP7B antibody in the 3 conditions. Lower panel: quantification of the phosphorylated protein at 160 kDa corrected for total protein, taking the control of each experiment (without calphostin C or PMA) as 100%. The data were analyzed using one-way ANOVA followed by Newman–Keuls post-test, and different lower-case letters above the bars indicate statistically significant differences ($p < 0.05$; $n = 12$).

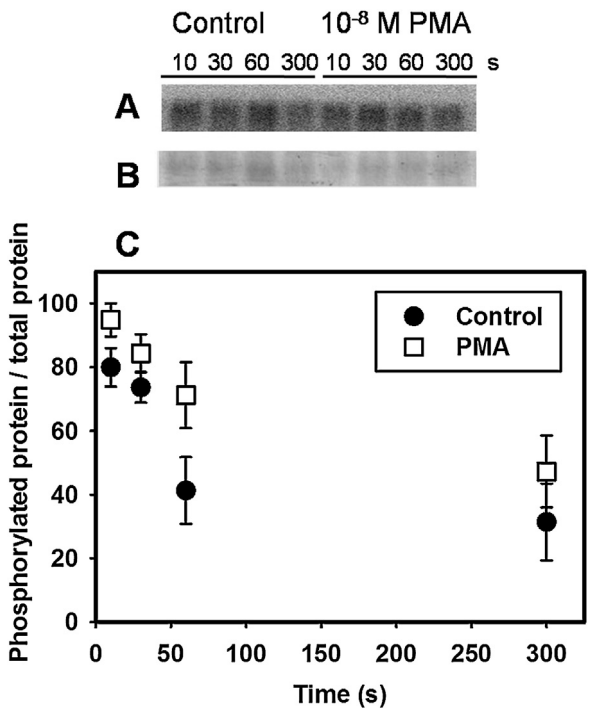

Fig. 11. PKC-mediated phosphorylation did not change the kinetics of phosphorylated intermediate decay formed during the ATP7B catalytic cycle. Catalytic phosphorylation of ATP7B was carried out in the absence (black circles) and presence (white squares) of 10^{-8} M PMA. Aliquots were removed at the indicated times on the abscissa. Data are mean ± SEM ($n = 4$) taking the maximal phosphorylation for each experiment as 100%. The same rate of decay is observed in both conditions, which were calculated using the exponential decay equation, $EP_t = EP_{max}\ (1 - e^{-kt})$, where k is the rate constant of phosphoenzyme decay. The values given in the text were calculated from independent fittings.

is not accelerated by ATP. Fig. 11 depicts the levels of phosphoenzyme in two conditions (control and with 10^{-8} M PMA) in all times tested. The levels of the phosphorylated intermediate were similar after 10 s in the absence or presence of PMA, and remained without statistical difference throughout phosphoenzyme decay. The single exponential decay gave similar rate constants in the absence or presence of PMA (0.0072 ± 0.0029 and $0.0081 \pm 0.0035\ s^{-1}$, respectively; $p > 0.05$). These data strongly suggest that the regulatory PKC-mediated phosphorylation should occur in a step after dephosphorylation of the copper pump, probably at the $E_2 \rightarrow E_1$ transition during catalysis (Lowe et al., 2004).

3.11. Alignment and prediction of consensus sites for PKC

Using two prediction tools (Scansite 2.0 and NetPhosK 1.0) and the Phosphosite database we analyzed different potential sites for PKC in the porcine sequence, taking into account the cytoplasmic loops (based in the human ATP7B) and solvent accessibility to select the 6 residues/sites shown in Table 1. However, the porcine ATP7B sequence is not completely assigned at the N- and C-terminus, according to the database UniProtKB. For this reason, we assessed the alignment of mammalian homologues looking for potential PKC target sites in these regions of ATP7B from human, mouse, rat, sheep and pig. A canonical sequence for PKCε (++XS/TX++; where: +, basic residue; X, any residue; S/T, Ser or Thr residue) was encountered in the C-terminus of four ATP7B sequences (Table 2). This consensus motif corresponds to Thr^{1317} in the human sequence, at the nucleotide binding domain just before the 7th transmembrane domain. Since the anti-ATP7B antibody used in Western blot (Fig. 10C) recognizes the C-terminus domain, it is clear that this region is present in porcine ATP7B. Altogether, these results are indicative that there are potential sites for PKC in porcine ATP7B and that the C-terminus of porcine ATP7B potentially contains the target sequence for PKCε.

Table 1
Predicted sites for PKC regulatory phosphorylation in porcine ATP7B sequence.

Residue	Score Scansite	Score NetPhosK	Sequence
Thr^{20}	0.44	0.71	PSQTASG**T**IRVGGMT
Ser^{754}	0.49	0.51	EALAKLM**S**LQATEAT
Thr^{761}	0.36	–	SLQATEA**T**VVTFGKD
Thr^{807}	0.48	0.51	GKVLEGS**T**MADESLI
Ser^{878}	0.49	0.51	QQLADRF**S**GYFVPFI
Thr^{980}	0.52	0.88	EMAHKIK**T**VMFDKTG

Table 2
Canonical sites for PKCε regulatory phosphorylation in mammalian ATP7B sequences.

Species	Sequence	Residue
Sheep (Q9XT50)	SRR**T**VWRI	Thr^{1357}
Mouse (Q64446)	SKR**T**VRRI	Thr^{1314}
Human (P35670)	SKR**T**VRRI	Thr^{1317}
Rat (Q64535)	SKR**T**VRRI	Thr^{1305}
Pig (A5A789)	--------[a]	–

[a] The C-terminus of the porcine ATP7B sequence is not yet available. A5A789 is at unreviewed state at UniProtKB/TrEMBL.

4. Discussion

In hepatocytes, ATP7B is normally found in *trans*-Golgi membranes, but under conditions of copper overload apical translocation of the pump is induced to allow transport of copper to the biliary capillaries. In a reversible process, the ATPase returns to the Golgi membranes when intracellular copper homeostasis has been restored (Guo et al., 2005). This recycling, crucial for copper excretion from the body, seems to rely on kinase-mediated mechanisms (Vanderwerf et al., 2001; Hasan et al., 2012). Although the entire repertoire of kinases regulating Cu(I)–ATPases trafficking is unknown, highly conserved serine residues that are potential targets for PKC and PKD – among other kinases – have been identified in both ATP7A and ATP7B (Veldhuis et al., 2009; Pilankatta et al., 2009, 2011). Catalysis by ATP7B itself is also regulated by kinase-mediated phosphorylations (Pilankatta et al., 2009; Hilário-Souza et al., 2011). An ensemble of interacting kinase signaling pathways is thought to be involved in regulating ATP7B during early steps of catalysis and in coupling catalytic phosphorylation to copper translocation through intramolecular and inter-domain communications (Liu et al., 2010). We previously showed that ATP7B is inhibited by PKA (Hilário-Souza et al., 2011), and we have now demonstrate that another protein kinase modulates Cu(I)-ATPase activity, an important step toward a better understanding of ATP7B function.

Stimulation of ATP7B activity in the presence of exogenous PMA indicates that mammalian hepatic tissue contains a membrane-associated DAG-dependent PKC isoform functionally coupled to the copper-transporting ATPase. The basal activity of ATP7B within its natural membrane environment is already significantly stimulated by PKC (in the absence of exogenous activators), since it inhibited by calphostin C. The hypothesis of a constitutive activating phosphorylation status is supported by phosphatase λ reducing ATP7B activity to a very low level in the absence of PMA. The phosphatase λ-induced drop of activity after PMA stimulation to the same level encountered without PMA further support the view that activation of the copper pump is the result of a PKC-mediated regulatory phosphorylation.

Opposing effects of PKC and PKA seem to constitute a paradigm for catalysis beyond ATP7B, since the recently-cloned ouabain-insensitive renal Na^+-ATPase (Rocafull et al., 2012), which is important in the fine-tuning of Na^+ transport (Bełtowski et al., 2007), has the same pattern of regulation (Rangel et al., 2005; Lara et al., 2010), which involves other enzymes in their respective cascades. Inhibition of Cu(I)–ATPase by U73122 shows the presence of an active PLC in Golgi liver membranes, indicating that any signal activating the PLC → PKC pathway can increase ATP7B activity. The presence of different PKC isoforms also points for a possible physiological role of signaling pathways mediated by protein G-coupled receptors, which might be triggered by hormones and other regulatory molecules in response to local copper fluctuations and binding of the metal to its enzyme sites (Vanderwerf et al., 2001; Vanderwerf and Lutsenko, 2002). Thus, it is tempting to propose that PKCs have a central role in the kinase-mediated phosphorylations that elicit responses to control local levels of copper by ATP7B transport activity and, ultimately, the subcellular localization of the pump (Vanderwerf et al., 2001; Hasan et al., 2012).

Ca^{2+} is a widespread intracellular messenger that exerts its influence by activating the classical PKC isoforms (Newton and Messing, 2010). *In vitro* studies show that different hormones, e.g. vasopressin, angiotensin II, noradrenaline and glucagon (Pittner and Fain, 1991) can induce DAG and Ca^{2+} release in hepatocytes, which could stimulate conventional and novel PKCs. The experiments using Ca^{2+} and PMA alone or together indicate that signaling pathways leading to Ca^{2+} release from intracellular stores are not coupled to processes involved in the stimulation of ATP7B activity, at least not in the liver. Since the inhibition promoted by the specific inhibitor peptide iPKCε (but not by iPKCζ) is comparable to that with calphostin C or phosphatase λ, and since PKCλ was not detected, it is reasonable to propose that PKCε is the only PKC isoform responsible for activating ATP7B in its natural environment. The leading role of PKCε in regulating hepatic copper handling is especially interesting in view of the uniqueness of this isoform in having Golgi binding domains, as also in interacting with proteins involved in Golgi budding and vesicular trafficking (Lehel et al., 1995; Csukai et al., 1997). Thus, the regulatory PKCε-mediated phosphorylation coupled to modulation of ATP7B-mediated copper transport seems to be coordinated with forward and retrograde trafficking of ATP7B to the Golgi network, a copper-induced subcellular redistribution (Vanderwerf et al., 2001). The small increase in regulatory phosphorylation after PMA that promotes a huge increment of Cu(I)–ATPase activity is indicative of a very sensitive and specific regulatory mechanism involving residues undergoing phosphorylation that may be amplified through long-range intramolecular communications during catalysis (Pilankatta et al., 2009). The small – though significant – inhibition of the regulatory phosphorylation of ATP7B by calphostin C, accompanied by a greater inhibition of ATP7B activity, reinforces the specific regulation of copper transport by PKCε. The results reflect a signal amplification mechanism, one of the pillars of signaling pathways process: the phosphorylation of a few residues could achieve a robust and specific signal transduction (Ubersax and Ferrell, 2007), which, in the present work, results in 50% PKC-mediated stimulation of ATP7B activity.

The molecular mechanism triggered by PKCε seems to accelerate the turnover of ATP7B, since the affinities for copper and ATP phosphoenzyme decay were unchanged in the presence of inhibitor or activator of PKC. Thus it is proposed that PKCε is responsible for a direct regulatory phosphorylation of ATP7B activating a catalytical cycle step after dephosphorylation of the enzyme, i.e., it is reasonable that PKCε accelerates the passage of unphosphorylated enzyme from the E_2 to the E_1 conformation before copper binding. This view is supported by the observation that the decay of the catalytic phosphorylated intermediate is not modified by activation of PKC by PMA at a low micromolar ATP concentration, i.e. when the rate limiting step of the cycle (the $E_2 \rightarrow E_1$ transition) is not accelerated by ATP.

The alignment analysis and prediction of PKC-target sites – using different neural networks – in porcine, human, mouse, rat and sheep ATP7B sequences showed several consensus motifs for PKC recognition, which provides additional evidence of a PKC-mediated regulatory phosphorylation of ATP7B. Besides, the existence of a highly conserved potential site for PKCε phosphorylation in the C-terminus of different mammalian ATP7B sequences – together with the inhibitory effect of iPCKε – suggests that this sequence might also be present in the porcine protein, giving further support to the idea of an isoform-specific PKC signaling mechanism. This site is located in the large cytoplasmic loop, between the nucleotide binding domain and the 7^{th} transmembrane motif, in the core of the protein. Thus, it can be proposed that phosphorylation of this Thr residue might lead to changes in the protein conformation, which results in a faster $E_2 \rightarrow E_1$ transition and accelerated overall turnover. Further studies using a site-directed mutagenesis approach should be performed to confirm this prediction, as we previously have done for Ccc2, the yeast copper ATPase (Valverde et al., 2008, 2011).

It might be that the regulatory role of PKCε demonstrated here points to a relationship with other kinases, as well as the complex network of cellular processes in liver that can be influenced by local copper levels under physiological and pathological conditions. Besides increases of DAG levels, the main effect triggered by glucagon is the stimulation of the PKA-mediated signaling

pathway (Li and Zhuo, 2007), which downregulates ATP7B activity (Hilário-Souza et al., 2011). Thus, PKCε seems to be the partner of PKA in regulating intracellular copper levels which in turn affects the balance among a wide repertoire of kinase-mediated phosphorylations, as proposed by Kaplan and Lutsenko (2009), together with modifications of key metabolic pathways. These cross-talks have important physiological and pathological manifestations: (i) hypoinsulinemic rats have high copper content in their hepatic cells, the underlying molecular mechanism not yet being understood (Failla and Kiser, 1983); (ii) PKCε interferes with insulin-regulated hepatic carbohydrate and lipid metabolism (Schmitz-Peiffer et al., 1997; Samuel et al., 2007). Unraveling of the effect of PKCε on ATP7B-mediated copper handling should help elucidate these complex interacting signaling pathways, which could be relevant in understanding the pathogenesis and possible treatment of Wilson disease. Some deleterious gene mutations could be overcome, at least partially, through manipulation of the kinase-mediated phosphorylations where PKCε has a central role.

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